Silencing Polyglutamine Degeneration with RNAi

Minireview

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Nine dominantly inherited neurodegenerative diseases are caused by expansion of a CAG repeat encoding glutamine. An important development in the study of such "polyglutamine" diseases was the realization that merely shutting off expression of a disease-encoding transgene could arrest progression in animal models with significant disease pathology. Such studies opened the door to a powerful new therapeutic approach now being pioneered: silencing of the dominant disease allele by RNA-mediated interference (RNAi), for the arrest—and potential reversal—of the disease process.

At least nine human neurodegenerative diseases are caused by CAG repeat expansions that encode extended runs of the amino acid glutamine (Gusella and MacDonald, 2000; Zoghbi and Orr, 2000). In addition to Huntington's disease (HD)—the most common and well-known disorder of this group—dentatorubral-pallidoluysian atrophy (DRPLA), spinobulbar muscular atrophy (SBMA), and six spinocerebellar ataxias (SCA1, -2, -3, -6, -7, and -17) comprise the polyglutamine (polyQ) diseases. PolyQ disease patients display a range of neurological phenotypes, with most suffering from progressive movement disorder and succumbing to disease within 10–20 years of onset; they typically share the same hallmark genetic mutation—expansion of a polymorphic polyQ repeat to beyond a threshold of ~37–40 glutamines. A fascinating aspect of the CAG/polyQ diseases, as well as other dynamic repeat disorders, including fragile X syndrome and myotonic dystrophy, is the correlation between repeat size and disease severity, such that the longer the repeat, the earlier the onset and more rapidly progressive the disease.

The expanded polyQ tract produces neuronal dysfunction and degeneration through abnormal protein interactions elicited by the formation of misfolded conformers. Indeed, an important breakthrough in this field was the observation that the mutant polyQ disease protein accumulates, culminating in the formation of inclusions visible at the light-microscopic level. Ubiquitination, and incorporation of various chaperones and proteasome components into these inclusions, likely reflects a decreased ability of the protein degradation machinery to efficiently turn over the protein (Muchowski and Wacker, 2005; Sherman and Goldberg, 2001). Additional proteins, including transcription factors and coregulators, are localized to inclusions, suggesting that sequestration of such cellular factors by the pathogenic protein may lead to loss of these activities and contribute to disease. To date, the most potent modulators of disease progression in animals have enhanced turnover or solubility of the polyQ protein through upregulation of chaperones, while other effective interventions have compensated for various downstream effects, including restoration of histone deacetylase activity (Di Prospero and Fischbeck, 2005).

RNA-Mediated Interference: The New Kid on the Block

Although such approaches offer promise, the most direct solution to counter polyQ disease pathogenesis is obvious: reduce expression of the mutant allele itself! Two compelling in vivo studies strongly support the potential success of such an approach (Yamamoto et al., 2000; Zu et al., 2004). In both, the respective teams of investigators generated conditional transgenic mouse models (one of HD and the other of SCA1) and demonstrated that shutting off expression of the mutant transgene dramatically slows disease progression and, for select features, even reverses severe disease pathology.

In 1998, studies performed with double-stranded RNAs in the nematode *Caenorhabditis elegans* revealed a sequence-specific RNA-mediated pathway for turning off gene expression (Zamore and Haley, 2005). The existence of this process, first observed in plants and now known as RNA-mediated interference (RNAi), across virtually all eukaryotic species soon became evident. Until this discovery, effective, targeted, and long-lasting suppression of a gene of interest was difficult and variable. However, with the adaptation of RNAi for use in mammals through the introduction of short-interfering RNAs (siRNAs) and short-hairpin RNAs (shRNAs), the immense potential of this technique as a therapeutic approach to treat dominant diseases has become apparent.

Dramatic Effects in Polyglutamine Disease Models Reveal RNAi's Therapeutic Potential

As the polyQ diseases involve a dominant gain-of-function effect of the polyQ expansion tract, numerous transgenic mice have been generated by simply expressing a human mutant protein in relevant neuronal populations with heterologous promoters. Such polyQ models provide an outstanding testing ground for the feasibility of the silencing approach: the first in vivo evaluation of RNAi as a treatment for dominant neurodegenerative disorders employed the very first polyQ disease mouse model—the SCA1 transgenic mouse. In this study led by the group of Beverly Davidson (Xia et al., 2004), a practical blueprint for the application of RNAi as a therapeutic intervention was outlined. The strategy consisted of two main parts. The first order of business was to identify an optimal oligonucleotide for robust knock-down of the target gene. As effective algorithms for oligonucleotide design were not refined at the time, a screen of randomly

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selected sequences was employed. From these studies in cell culture emerged a potent ataxin-1 shRNA for the job. The second critical component of the RNAi therapeutic approach is mode of delivery. As the SCA1 transgenic mice display restricted expression of mutant ataxin-1 to Purkinje cells (postmitotic neurons of the cerebellum), the Davidson group evaluated adeno-associated virus serotype 1 (AAV1) for its ability to successfully transduce these neurons. They then derived an AAV1 ataxin-1-shRNA construct, generated high-titer AAV preparations, and injected the shRNA-expressing viruses into the cerebella of 7-week-old SCA1-82Q transgenic mice. The results were striking. They observed significant improvements in treated mice within 2 months of viral injection for motor coordination and Purkinje cell neuropathology. Especially compelling was a dramatic diminution in the accumulation of the pathogenic ataxin-1 82Q protein in the nuclei of Purkinje cells from transduced folia of treated mice.

Next, the Davidson group turned their attention to the most common polyQ disease, HD (Harper et al., 2005). The approach was similar—first identifying an effective shRNA by screening candidates in cell culture cotransfections and then confirming that AAV1 shRNAs injected into striatum would infect those cells. They then proceeded to inject AAV1-huntingtin (htt) shRNAs into the striata of 4-week-old HD transgenic mice. The N171-82Q HD mouse model was selected because this truncation fragment model contains enough of the htt reading frame to permit targeting by a specific shRNA, and the model displays a relatively rapid disease course, reaching end stage by 5–6 months. In treated mice, dramatic reductions in soluble htt protein were observed as rapidly as 2 weeks after viral injection, with reductions in inclusions at end stage in the striatum and total elimination of cerebellar inclusions upon injection there. While rotarod performance was nearly normalized in treated mice, stride length improved but remained impaired relative to controls, indicating a less pronounced effect of striatal delivery upon this behavioral trait. Striatal delivery did not prevent weight loss in treated HD mice, and its effect upon survival was not measured. When one considers the widespread expression pattern of prion protein promoter driven htt in the N171-82Q mice, however, the ability of striatal-delivered htt shRNA to markedly improve two key motor deficits is remarkably encouraging.

Limitations and Challenges: Hurdles to Be Cleared Before Going to the Clinic

While the SCA1 and HD studies highlight the enticing therapeutic potential of RNAi, application of this strategy to human patients may not be so straightforward. An obvious difference between these mice and human patients is that SCA1 and HD transgenic mice possess two copies of the corresponding endogenous genes. As RNAi knockdown was directed selectively to the human polyQ transgene in these experiments, simultaneous knockdown of the normal endogenous ortholog of the disease gene was avoided. But in the human disease situation, the shRNAs being used would reduce expression of both the normal and disease alleles. Although evidence for a dominant gain-of-function effect with polyQ expansion is overwhelming, strong data also suggest that a concomitant partial loss of normal function occurs for a number of polyQ disease proteins. Notably, huntingtin may be involved in neurotrophic factor transcription or transport and/or may function in an antiapoptotic capacity, with striatal neurodegeneration occurring when expression is eliminated in mice postnatally (Dragatsis et al., 2000). Partial loss-of-function accounts for androgen insensitivity in SBMA (Katsuno et al., 2004), and suppression of ataxin-7 could have deleterious effects upon the transcription coactivator complex to which it belongs (Palhan et al., 2005). Similarly, normal ataxin-3 may play a role in mitigating accumulation of misfolded proteins in polyQ and other neurodegenerative diseases (Warrick et al., 2005). Thus, while elimination of select polyQ disease proteins in knockout mice yields no dramatic untoward effects (such as ataxin-1), knock-down of other polyQ disease proteins could produce a whole other set of unacceptable pathologies.

Although partial reduction of gene expression may be well tolerated, an ideal solution to this problem would be to target shRNA constructs selectively to the mutant allele. As moderately long CAG tracts are present in numerous genes, let alone in the normal allele corresponding to the disease gene of interest, repeat-specific oligonucleotides are not feasible. A plausible approach, however, is to take advantage of occasional linked polymorphisms in the disease allele and to construct shRNAs directed to those regions. Indeed, only a single nucleotide polymorphism (SNP) is sufficient for allele-specific targeting with RNAi. In SCA3, expanded CAG repeat tracts are always followed by a cytosine in a wide range of population groups, while a guanine typically follows the CAG repeat tract in normal alleles (Limprasert et al., 1996). Consequently, many SCA3 patients will possess versions of the ataxin-3 gene whose coding regions will vary between the disease and normal alleles, offering the prospect of allele-specific knock-down (Li et al., 2004). Whether such common linked polymorphisms can be ferreted out for other polyQ disease loci remains to be seen. However, as coding region SNPs do occur with considerable frequency, one could envision haplotyping strategies aimed at the detection of disease allele-specific SNPs on a patient-by-patient basis, followed by selection of the matching knock-down construct from a set of validated shRNAs. It may be possible, for example, to design a set of 16 shRNA knockdown constructs for a single locus in the hope that haplotype screening of merely 8 SNPs per patient will yield at least one disease allele-linked SNP to permit selective targeting.

Even if these approaches prove problematic, global deleterious effects could be circumvented by restricting therapeutics to select cell types or brain structures central to disease symptoms (e.g., cerebellum for the SCAs, striatum for HD). Notably, in studies of RNAi targeting to silence the pathogenic SCA1 transgene, disease progression was mitigated with only partial viral infection of the cerebellum. Cell-type and region-specific treatment could be a practical advantage of the RNAi approach, as widespread delivery may not be required for marked improvement. On the other hand, this could also be a limitation: if one can only treat select areas, then degeneration in other regions may progress unabated. And as non-cell-autonomous degeneration

may be operating in a number of neurodegenerative disease processes including polyQ disorders, such restrictions could undermine therapeutic efficacy.

Critical for the success of the RNAi approach is the availability of safe, precise, and robust methods of delivery to the central nervous system (CNS). Over the last decade, significant advances in viral-vector development and design have yielded promising options for CNS treatment (Davidson and Breakefield, 2003), with most of the non-cancer-related CNS gene therapy work focused upon lentivirus and AAV. Recombinant AAV has emerged as an appealing system for gene delivery due to development of improved vectors and purification systems, as well as the discovery of various serotypes with high rates of infectivity. In the SCA1 and HD work, the Davidson group selected AAV, as such viral vectors can be maintained for many months in neurons and tend not to integrate into the host genome—such integration, as occurs with lentivirus, could present added problems of disrupting endogenous gene activity. One important issue is host cell range, as the virus must successfully infect the appropriate neurons, while another issue is length of treatment. As AAV tends not to integrate, but rather exists episomally, repeated treatment may be required. The integration property of lentivirus could be an advantage in this regard.

Other ingredients central to success include how to control shRNA expression and which cells to target. Once studies have established the least extensive host cell range for therapeutic efficacy, promoters that drive restrictive patterns of gene expression may be preferable, due to concern for off-target effects. Potential off-target effects remain a serious issue for any shRNA therapy, as experience with RNAi as a therapeutic intervention is limited to date. Conditional promoters—which may be essential clinically for an approach as potentially powerful as RNAi knockdown—would allow titration of the extent of gene-silencing for exquisite control: modest knock-down may be sufficient for less severe diseases or early intervention, whereas more robust silencing might be required for advanced disease or later time points. A drawback is that such promoters may present their own risks by requiring expression of a foreign transcriptional protein or long-term treatment with a regulator drug. There is also the issue of timing: RNAi knockdown may be powerful at early stages, but once downstream events have played out, targeting the disease gene itself may no longer be therapeutically beneficial. Of course the ultimate stumbling block to successful implementation of the RNAi approach for neurological diseases is delivery. Hitting the target CNS region is by no means trivial and will require stereotactic surgical procedures and/or catheter placement, manipulations whose success will depend upon the specific region being targeted. Nonviral RNAi methods are also in development (Dorsett and Tuschl, 2004), with similar issues of delivery and specificity, although the use of osmotic pumps with such approaches is particularly appealing due to the ability to readily control the therapeutic (Vande Velde and Cleveland, 2005). Ultimately, the clinical feasibility of any RNAi therapy will hinge upon our ability to overcome all of these issues.

Therapeutic Opportunities in Neurodegenerative Disease Beyond PolyQ Disorders

The power of the RNAi approach is now being applied to other neurological disorders, including amyotrophic

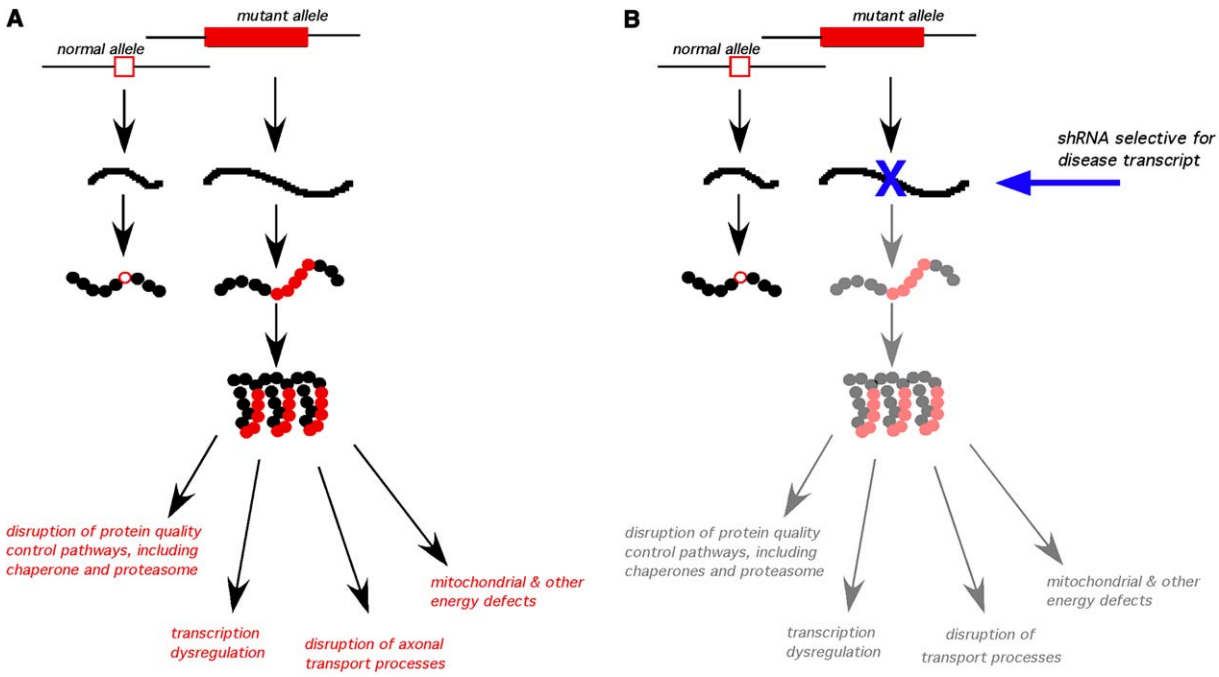

Figure 1. RNAi Therapy for Dominant Neurological Disease: Polyglutamine Disorders as an Example

(A) Mutant protein, expressed from the mutant allele, bears an abnormally long polyQ domain, resulting in protein accumulation triggering a myriad of deleterious events, culminating in neuronal dysfunction and loss. (B) Treatment with virus expressing shRNA selective for the mutant allele knocks down levels of the mutant transcript, reducing mutant protein production and theoretically alleviating all resultant deleterious events.

lateral sclerosis (ALS) and Alzheimer's disease (AD). The vast majority of AD cases are sporadic—so what gene do you target? A potentially ideal target is β-secretase (BACE)—an enzyme that helps cleave amyloid precursor protein (APP), contributing to amyloid plaque formation associated with degenerative changes. Complete knock-out of BACE function in the brains of mice has minimal effects, implying that targeting it with RNAi should be highly tolerable. Recent results indicate that intracerebral injection of BACE shRNA lentivirus vectors into an APP transgenic mouse model of AD yielded reduced amyloid deposition in the hippocampus and neocortex, with full recovery of spatial learning and memory (Singer et al., 2005). In ALS, intramuscular delivery with equine infectious anemia virus (EIAV) yielded an impressive 80% extension in survival for the SOD1 mouse model of inherited ALS1—the longest therapeutic mitigation to date (Ralph et al., 2005), suggesting that this lentiviral delivery method may be promising for all motor neuron diseases. Although application of RNAi to sporadic ALS suffers from the lack of a clear target, extending the approach to sporadic Parkinson's disease (PD) by targeting α-synuclein could be promising, because α-synuclein expression levels appear to directly correlate with disease risk. Such work is likely already in progress.

Closing Thoughts

Despite the theoretical complications and practical problems facing RNAi therapy for CNS disease, it is now abundantly clear that the approach holds tremendous promise. For polyQ diseases, one envisions that the mutant protein is the trigger for an ever-increasing cascade of diverse problems that affect a wide range of fundamental cellular processes (Figure 1A). It is reasonable to expect that maximal therapeutic benefit will be achieved by directing treatment as close as possible to the origin of the disease process—that is, close to expression of the mutant protein for such dominant neurodegenerative disorders (Figure 1B). A key question is: can RNAi knock-down provide significant therapeutic benefit after downstream deleterious effects have been triggered? This is particularly crucial, because patients with most neurodegenerative diseases do not present to clinic until significant underlying pathology already exists. In the case of the polyQ diseases, however, there is a unique opportunity to circumvent this problem for "presymptomatic" patients, as such at-risk individuals can be identified prior to disease onset with genetic testing. For other patients, a combinatorial approach—which hits multiple points from initiating events to downstream consequences—may prove most efficacious.

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